6

Microbiology of yoghurt and "bio" starter cultures

6.1 Introduction

The first bacteriological study of yoghurt was made by Grigoroff (1905) who observed three different micro-organisms present, namely a diplostreptococcus, a rod/coccal-shaped *Lactobacillus* and a rod-shaped *Lactobacillus.* The same observation was also reported by Lüerssen and Kühn (1908). However, the popularity of yoghurt could be attributed to Metchnikoff (1910), who postulated the theory that prolongation of life would follow ingestion of a lactic acid bacterium named as *Bulgarian bacillus.* The presence of this organism in yoghurt was supposed to inhibit the growth of putrefactive organisms in the intestine.

The *Bulgarian bacillus* is, in fact, *Thermobacterium bulgaricum* (Orla-Jensen, 1931), later designated as *Lactobacillus bulgaricus* (currently known as *L. delbrueckii* subsp. *bulgaricus*). However, Rettger and Cheplin (1921) and Rettger *et al.* (1935) found that *Thermobacterium acidophilin (Lactobacillus acidophillus)* is the lactic acid bacterium that can establish itself in the intestine, and furthermore, that the main therapeutic value of yoghurt is observed when *L. acidophilus* is one of the bacteria present in the starter culture. The classification of the lactic acid bacteria by Orla-Jensen (1931) is still recognised as the standard method for distinguishing these organisms, i.e. the sphere shape was *Streptococcus* and the rod forms were *Thermobacterium*, *Streptobacterium* and *Betabacterium.* According to Orla-Jensen (1931), the yoghurt starter organisms were thermophilic lactic acid bacteria capable of growing at 40–45°C. These organisms were designated as *Thermobacterium bulgaricum*, *Thermobacterium jugurti* (*Lactobacillus jugurti*) and *Streptococcus thermophilus.* According to the seventh edition of Bergey's Manual (1957), all the lactic acid bacteria were grouped into one family, the Lactobacillacceae, which was subdivided into the Streptococceae (ovoid or spherical in shape) and the Lactobacilleae (rod-shaped). But this classification was reorganised in the eighth edition of Bergey's Manual (1974) to give two separate families, the Streptococcaceae and the Lactobacillaceae, whilst in the latest edition of Bergey's Manual (1986) the same

organisms are grouped in different sections. For example, the Gram-positive cocci consist of two families where the genus *Streptococcus* is grouped in family II, i.e. Deinococcaceae. However, the genus *Lactobacillus* is grouped in a separate section known as regular, non-sporing, Gram-positive rods. The group-N lactic streptococci (i.e. the mesophilic type) are now known as *Lactococcus* species, and *S. thermophilus* (i.e. a thermophilic organism) has retained its nomenclature.

6.1.1 Historical background and classification

The taxonomic status of *S. thermophilus* reported by Orla-Jensen (1931) has fluctuated since the 1980s due to the close relationship between this organism and *Streptococcus salivarius* and, as a consequence, it was denoted as a subspecies (e.g. *S. salivarius* subsp. *thermophilus*). In 1991, a separate species status was reproposed on the basis of both genetic and phenetic criteria; for further detail see the reviews by Hardie and Whiley (1992, 1995). Selected characteristics of *S. thermophilus* are shown in Table 6.1. Other characteristics may include:

- Spherical or ovoid cell morphology, $<1\,\mu m$ in diameter and forming chains or occurring in pairs.
- Absence of growth at 15°C, whilst growth at 45°C may give rise to irregular cells and segments; most strains are able to grow at 50°C or survive heating for 30 min at 60°C.
- Bacteria are Gram-positive, anaerobic homofermentative lactic acid and produce L(+) lactate, acetaldehyde and diacetyl from lactose in milk.
- Some strains produce exopolysaccharide (EPS), and require B vitamins and some amino acids for enhanced growth rates.
- Absence of growth in methylene blue ($0.1\,g\,100\,ml^{-1}$) or at pH 9.6.
- The cell wall peptidoglycon type is Lys-Ala_{2-3}, and 16S rRNA sequence data have demonstrated close association between *S. thermophilus*, *S. salivarius* and *Streptococcus vestibularis*.
- A group antigen for serological identification has not been demonstrated (see also Nour *et al*., 1989; Ehrman *et al*., 1992).

The situation is different when certain *Lactobacillus* spp. are considered with regard to classification and nomenclature. The standard method proposed by Orla-Jensen (1931) (i.e. *Thermobacterium*, *Streptobacterium* and *Betabacterium*) has been replaced using group I, II or III in the latest edition of Bergey's Manual (1986); however, the history of the group and the redefinitions of the lactobacilli have been reviewed by Bottazzi (1988), Collins *et al*. (1991), Hammes *et al*. (1992), Hertel *et al*. (1993), Pot *et al*. (1994) and Hammes and Vogel (1995). Studies of the guanine plus cytosine (G + C) content of deoxyribonucleic acid (DNA), DNA–DNA hybridisation and enzyme homology have shown that *L. jugurti* is a biotype of *Lactobacillus helveticus* and there is no reassociation between *L. bulgaricus* and *L. jugurti* (Simonds *et al*., 1971; Nakamura and Anzai, 1971). The DNA homology between *L. jugurti* and *L. helveticus* is about 80–100%, and the former which is considered to be a maltose-negative variant of *L. helveticus* is not recognised any more (London, 1976). However, because of the high phenotypic and genomic similarities between *Lactobacillus delbrueckii*, *leichmanni*, *lactis* and *bulgaricus*, only *L. delbrueckii* has been retained as a separate species, whilst the other organisms are subspecies. Both *L. lactis* and *L. leichmanni* are grouped as *L. delbrueckii* subsp. *lactis* and

Table 6.1 Selected characteristics of some lactic acid bacteria[a] associated with yoghurt

Characteristic	*Streptococcus* spp.		*L. delbrueckii* subsp.			*Lactobacillus* spp.		
	thermophilus	*salivarius*	*delbrueckii*	*bulgaricus*	*lactis*	*acidophilus*	*helveticus*	*jugurti*[b]
G + C[c] mean (%)	37–40	39–42	49–51	49–51	49–51	34–37	38–40	39
Lactic acid isomer(s)	L(+)	L(+)	D(−)	D(−)	D(−)	DL	DL	DL
Growth at 10/45°C	−/+	−/+	−/+	−/+	−/+	−/+	−/+	−/v
Requirement for								
Thiamine			−	−	−	−	−	−
Riboflavine			+	+	+	+	+	+
Pyridoxal			−	−	−	−	+	+
Folic acid			+	+	+	+	−	−
Thymidine			+	+	+	−	−	−
Vit. B_{12}			+	+	+	−	−	−
Carbohydrate utilisation								
Aesculin	+	+	−	−	+	+	−	−
Amygladin	+	−	−	−	+	+	−	−
Cellobiose	+	−	d	−	d	+	−	−
Fructose	+	+	+	+	+	+	d	
Galactose	−	d	−	−	d	+	+	+
Lactose	+	+	−	+	+	+	+	+
Maltose	+	−	d	−	+	+	d	−
Mannose			+	−	+	+	d	
Melezitose			−	−	−	−	−	
Melibiose	−	d	−	−	−	d	−	−
Raffinose	−	d	−	−	−	d	−	
Ribose	−	d	−	−	−	−	−	
Salicin	+	−	−	−	+	+	−	−
Sucrose			+	−	+	+	−	−
Trehalose	d	−	d	−	+	d	d	d

[a] None of the organisms produce gas from gluconate and glucose or NH_3 from arginine. [b] *L. jugurti* is included for comparative purposes. [c] Mean % of guanine and cytosine of DNA.

+, Positive reaction by 90% or more strains; −, negative reaction by 90% or more strains; d, positive or weak reaction by 11–89%;, empty spaces indicate no data available.

Data compiled from Hansen (1968), Rogosa and Hansen (1971), Bergey's Manual (1974, 1986), Ottogalli *et al*. (1979), Accolas *et al*. (1980), Tamime (1990), Hammes and Vogel (1995) and Hardie and Whiley (1995).

L. bulgaricus is currently known as *L. delbrueckii* subsp. *bulgaricus*. Table 6.1 illustrates the overall differences between these various lactobacilli. Other characteristics of *L. delbrueckii* subsp. *bulgaricus* are:

- It is represented in Group I or Aa – the obligately homofermentative lactobacilli; the letter a indicates the affiliation to the *L. delbrueckii* group.
- The cells are rods and rounded ends, of 0.5–0.8 × 2–9 μm, and occur singly or in short chains.
- This organism ferments fewer sugars, produces D(+) lactate and acetaldehyde from lactose in milk, and some strains produce EPS.
- Slight growth occurs at <10°C and most strains are able to grow at 50–55°C.
- The cell wall peptidoglycan type is Lys-DAsp (see also Park *et al*., 1991; Sungil *et al*., 1996).

In view of the wide range of technical data available on yoghurt and other dairy starter cultures, it is recommended that the reader consults some selected publications for general information (Accolas and Auclair, 1983; Auclair and Accolas, 1983; Bianchi-Salvadori, 1983; Sriranganathan *et al*., 1985; Chassy, 1986; Terre, 1986; Marshall, 1986, 1987, 1993; Kashket, 1987; Daly, 1987; IDF, 1988a; Roginski, 1988; Lücke *et al*., 1990; Schleifer *et al*., 1991; Gasser, 1994; Roussis, 1994; Stiles, 1996).

According to Mitsuoka (1992), *L. acidophilus* was first isolated from faeces of bottle-fed infants and named *Bacillus acidophilus*, but in 1959, Rogosa and Sharpe gave a detailed description of *L. acidophilus* based on their own observations and those of Tittsler *et al*. (1947) and Rogosa *et al*. (1953). Later, Lerche and Reuter (1962) subdivided the species into five biotypes based on fermentation patterns of trehalose, melibose and raffinose, while Mitsuoka (1969) expanded the number of biotypes to ten based on variations in the fermentation of ribose and lactose. More recently, the phylogenetic approach based on 16S rRNA adopted by Collins *et al*. (1991) and Fujisawa *et al*. (1992) has cast doubt on some of these earlier groupings but, even so, the identity of *L. acidophilus* as proposed by Gasser and Mandel (1968) remains intact. As a consequence, the description of the species by Hansen and Mocquot (1970) based on a specific strain (ATCC 4356) is still valid.

The taxonomic status of *L. acidophilus* has not fluctuated over the years and whilst some characteristics of this organism are shown in Table 6.1, some other aspects may include:

- It is presented in Group I or Aa – the obligately homofermentative lactobacilli; in the same group as *L. delbrueckii* subsp. *bulgaricus*.
- The cells are rods with rounded ends, of 0.6–0.9 × 1.5–6 μm, occurring singly, in pairs and in short chains; cells are non-motile and non-sporulating and proteins in the cell wall may be important in attaching the bacterium to the intestinal wall (Bhowmik *et al*., 1985; Brennan *et al*., 1986).
- This organism requires riboflavin, pantothenic acid, folic acid and niacin for growth, but not the other B vitamins.
- Recent studies (i.e. electrophoresis of cellular proteins or lactate dehydrogenase and DNA–DNA reassociation) suggest that *L. acidophilus* strains include six genomospecies.
- No growth occurs at <15°C, most strains grow about 35–45°C and the optimum pH for growth is 5.5–6.0.
- The cell wall peptidoglycan type is Lys-DAsp.

According to Mital and Garg (1992), the growth requirements of most strains of *L. acidophilus* are quite complex and, as the normal habitat of *L. acidophilus* is attached to the walls of the small intestine of mammals, such requirements can usually be met quite easily. The ability of the species to utilise carbohydrates *in vitro* is shown in Table 6.1 and although *L. acidophilus* is the best known of the health-promoting lactobacilli, other species of human intestinal origin are often used in fermented milk and comparable data for some of these species has been included as well. In addition, strains of *L. acidophilus* may require fatty acids, minerals, peptides and amino acids, nucleic acid derivatives and vitamins of the B-complex to grow successfully and, given these requirements, it is not surprising that most strains grow only poorly in bovine milk. The final value of lactic acid is within the range of 0.3–1.9 $g\,100\,g^{-1}$ lactic acid suggested by Rasic and Kurmann (1978) but, while some strains can secrete these high levels of acid, few strains are sufficiently acid tolerant to survive such conditions for more than a few days; the optical rotation of the lactic acid is DL.

The alleged health-promoting properties of *L. acidophilus* are discussed elsewhere and it is relevant that, in addition to secreting lactic acid, some strains of the species may produce antibiotic-like substances as well. Some authors have suggested that such compounds could be important in preventing the growth of pathogens in the intestine (Shahani *et al*., 1976), but it could be that intrageneric activity could be equally relevant. Thus, Barefoot and Klaenhammer (1983) and Barefoot *et al*. (1994a, b) purified a bacteriocin compound from a strain of *L. acidophilus* and found it to be active against a range of other *Lactobacillus* spp. If this inhibitory activity happens in the intestine as well, then it might provide an additional mechanism whereby indigenous strains of *L. acidophilus* could retain dominance on the epithelial surfaces.

However, the taxonomic and nomenclature situation of the bifidobacteria have changed, and in the eighth edition of Bergey's Manual (1974) they were classified as *Lactobacillus* spp., whilst in latest edition of Bergey's Manual (1986) the same organisms are grouped in a different section, and known as *Bifidobacterium* spp.

Currently, 30 different strains of bifidobacteria have been identified which have been isolated from different sources such as the faeces of humans, animals, birds and sewage, the human vagina, bees and dental caries. Only six species of bifidobacteria have attracted attention in the dairy industry for the manufacture "bio" ferment dairy products. These organisms are known as *Bifidobacterium adolescentis*, *breve*, *bifidum*, *infantis*, *lactis* and *longum*, and these species have been isolated from human subjects for the manufacture of fermented milk. This restriction is based on the assumption that, if an isolate is of human origin, then it should become implanted on the walls of, and/or metabolise in, the colon of another human. The validity of this idea remains open to debate, for there is some evidence that, while an ingested strain may dominate the colon walls of a patient with a low count, the strains that are indigenous to that patient will, in time, overgrow the invading culture. It is relevant also that non-human strains of *Bifidobacterium animalis* can adhere to human cells in tissue culture, so that the question of which species should be permitted in bio-yoghurts is a matter of some debate.

The differentiating characteristics have been reviewed in Bergey's Manual (1986) and by Biavati *et al*. (1992), Sgorbati *et al*. (1995), Tamime *et al*. (1995), Kok *et al*. (1996), Meile *et al*. (1997) and Ballongue (1998). Other characteristics may be considered.

- Bacteria are Gram-positive, anaerobic heterofermentative, non-motile, non-spore-forming rods (0.5–1.3 × 1.5–8 μm).
- Cell morphology of these bifidobacteria grown anaerobically in trypticase-phytone-yeast (TPY) medium have distinctive shapes and arrangements (e.g. "amphora-like"; specific epithet, thin and short; very elongated, thin with slight irregular contours and rare branching).
- The cell wall peptidoglycan varies among the species, but this complex material consists of linear chains of *N*-acetylmuramic acid and *N*-acetylglucosamine molecules alternating along the length of the chain.
- Different species utilise different types of carbohydrates (see Table 6.2) and such fermentations are used for identification purposes. One key enzyme involved is fructose-6-phosphate phosphoketolase (F6PPK) known as "bifidus shunt", and this enzyme can be used to identify the genus; it should be noted that not all strains produce enough F6PPK for it to be detectable. The fermentation of two molecules of glucose leads to two molecules of lactate and three molecules of acetate.
- The guanine plus cytosine molecular percentage of the DNA of this genus ranges between 54 and 67.
- A wide range of components have been identified as bifidogenic growth stimulators.

The rods of bifidobacteria often have an irregular shape, with a slightly concave central region and swollen ends (i.e. having the appearance of a dog's bone in a Disney cartoon). It is, however, not unusual to encounter cells that are coccoid or appear as very long or short bacilli of varying widths, or the cells may be V, Y or X-shaped depending on the constituents of the medium on which the colony is growing. It is believed that, under adverse growing conditions, the cell morphology changes to produce more branched cells; for example, in a medium deficient in β-methyl-D-glucosamine, the cells become more branched, while the addition of certain amino acids (e.g. serine, alanine or aspartic acid) can transform X- or Y-shaped cells into curved rods (Glick *et al.*, 1960). Similarly, Samona and Robinson (1994) transformed coccoid cells of *B. bifidum* into the Y-shaped form through the addition of sodium chloride to a medium, but noted that neither *B. longum* nor *B. adolescentis* reacted in the same way. The same authors recorded also that the pattern of carbohydrate fermentation changed as the morphology altered, suggesting perhaps that the permeability of the cell membrane to certain sugars was being modified in parallel with the structural changes taking place in the wall.

Notwithstanding this tendency of some species to alter in shape, the cell morphology of species of bifidobacteria grown anaerobically in stabs of TPY extract medium showed a tendency to adopt distinctive cellular shapes. For example, *B. bifidum* forms groups of amphora-like cells, the cells of *B. breve* are the thinnest and shortest among bifidobacteria, while *B. longum* appears as very elongated, relatively thin cells with slightly irregular contours.

A summary of cell wall and DNA contents of the important species of bifidobacteria species are shown in Table 6.2. The principal component of the cell wall is peptidoglycan, also known as murein. This is a macromolecule that consists of linear polysaccharide chains (glucose, galactose and rhamnose) which are linked to each other by tetrapeptide bridges (Ballongue, 1998).

Table 6.2 Some selected characteristics of bifobacteria used for the manufacture of bio-yoghurt[a]

Characteristic	*Bifidobacterium* spp.					
	adolescentis	*bifidum*	*breve*	*infantis*	*lactis*[b]	*longum*
G + C mean (%)	58.9	60.8	58.4	60.5	61.9	60.8
Type of peptidoglycan	Lys(Orn)-D-ASP	Orn(Lys)-D-Ser-D-ASP	Lys-Gly	Orn(Lys)-Ser-Ala-Thr-Ala	Lys(Orn)-Ala-(Ser)-Ala_2	Orn(Lys)-Ser-Ala-Thr-Ala
Carbohydrate utilisation						
Arabinose	+	–	–	–	d	+
Cellobiose	+	–	d	–	–	–
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Gluconate	+	–	–	–	–	–
Inulin	d	–	d	d	+	–
Lactose	+	+	+	+	+	+
Maltose	+	–	+	+	+	+
Mannitol	d	–	d	–	–	–
Mannose	d	–	+	d	–	d
Melezitose	+	–	d	–	–	+
Melibiose	–	d	–	+	+	+
Rafinose	+	–	+	+	+	+
Ribose	+	–	+	+	+	+
Salicin	+	–	+	–	–	–
Sorbitol	d	–	d	–	+	–
Starch	+	–	–	–	–	–
Sucrose	+	d	+	+	+	+
Trehalose	d	–	d	–	+	–
Xylose	+	–	–	d	d	d

[a] For identification of symbols see Table 6.1. [b] After Kok *et al.* (1996) and Meile *et al.* (1997).

Data compiled from Bergey's Manual (1986), Biavati *et al.* (1992) and Sgorbati *et al.* (1995).

6.1.2 Modification of starter cultures

The characteristics of the various species shown in Tables 6.1 and 6.2 are based essentially on what are referred to as type cultures. These are strains of the species that have been: (a) isolated and grown as pure cultures in one of the internationally recognised culture laboratories, (b) examined for a range of characteristics, such as temperature of growth and/or rate of acid production (Zanatta and Basso, 1992), fermentation of selected sugars (Hickey *et al*., 1986), enzyme profiles (Bianchi-Salvadori *et al*., 1995), DNA base-pair characteristics (Sriranganathan *et al*., 1985), DNA hybridisation reactions (Lick *et al*., 1996), plasmid homology and/or profiles (Girard *et al*., 1987; El-Soda *et al*., 1989) and DNA fingerprinting (Ramos and Harlander, 1990), and then (c) designated as a distinct species. This procedure means that there is held somewhere in a deep frozen (–196°C) or freeze-dried state, a culture which displays all the characteristics of one recognised species and, once these characteristics have been recorded in an authoritative reference source (e.g. Bergey's Manual, 1986) anyone in the dairy industry or elsewhere can identify, with a reasonable degree of certainty, any cultures that may be isolated from cheese or a fermented milk.

For many years, this approach to bacterial taxonomy has worked well, but since about 1990, the degree of strain variability within species has increased because taxonomists have begun to employ increasingly sophisticated techniques for identification, for example, 16S RNA sequencing (Davidson *et al*., 1996) and the use of DNA probes to isolate individual strains (Delley *et al*., 1990; Colmin *et al*., 1991; Neve and Soeding, 1997), and the number of cultures available from commercial suppliers has increased.

Some of this variability has arisen as a natural process of change, because the selective pressures on a culture of *S. thermophilus* employed in a dairy in the Middle East, for example, might well be different from those operational in a plant in North America (Nunez de Kairwuz *et al*., 1983; Yoast *et al*., 1994; Teixeira *et al*., 1994). The same species isolated from a cheese factory in Italy might well be different again, so that the precise definition of a species becomes, in some respects, more difficult (Sandine, 1987; Mercenier and Lemoine, 1989). A good example of this situation can be found for the mesophilic starters for cheese, in that while the type culture of *Lactococcus lactis* subsp. *cremoris* differs widely from *Lactococcus lactis* subsp. *lactis* with respect to the sugar fermentation pattern, a culture of *L. lactis* subsp. *cremoris* purchased today may well display the same sugar utilisation profile as *L. lactis* subsp. *lactis* (de Vos, 1996).

Although this complicated situation may, in part, be the result of culture evolution as a result of mutation (Mollet and Delley, 1990; see also Germond *et al*., 1995), conjugation (Kleinschmidt *et al*., 1993; Soeding *et al*., 1993), transformation (Mollet *et al*., 1993b) and intercellular and/or plasmid transduction (Mercenier *et al*., 1988a, b; Heller *et al*., 1995; Neve and Heller, 1995a, b), the deliberate genetic manipulation of cultures has become increasingly important (Yu *et al*., 1984; Chassy, 1987; Romero *et al*., 1987; Knol *et al*., 1993a, b; Sasaki, 1994; Mercenier *et al*., 1994). Thus, genetic engineering or recombinant DNA technology can now be employed to modify the properties of various organisms to generate genetically modified organisms (GMOs) (Herman and McKay, 1986; de Vos and Simons, 1988; Somkuti and Steinberg, 1988, 1991; Lee *et al*., 1990b; Gasson, 1997).

To avoid potential conflicts with consumers, bacteria to be used in the manufacture of foods should be subject only to so-called food grade genetic modifications,

which means that the GMO must contain only DNA from the same genus and, possibly, small stretches of imported DNA (Johansen *et al*., 1995). Thus, a *Lactococcus* GMO would only contain DNA from the genus *Lactococcus* plus a small amount of imported DNA (Mollet *et al*., 1993a; Griffen and Gasson, 1995). These small stretches of non-lactococcal DNA are usually no longer than 50 base pairs and act as recognition sites for the restriction enzymes used in the actual construction process (Solaiman and Somkuti, 1991, 1995, 1997a–c; Somkuti and Solaiman, 1997; Satoh *et al*., 1997). It is essential, of course, that none of the imported DNA should provide a code for RNA, and specific DNA probes should be constructed to check that no additional genetic material has been introduced (Lick and Teuber, 1992).

However, pressure is mounting within the dairy industry for permission to exchange DNA between any genus of micro-organism associated with food fermentation (Langella and Chopin, 1989), provided that the donor bacterium can be described as generally recognised as safe (GRAS). Whether or not it is appropriate for microbiologists to borrow this definition from the chemists has not been challenged, but it is relevant that food-grade GMOs can usually be used in the United States without specific regulatory approval.

6.1.3 Potential genetic modifications

Genes can be deleted from a strain to avoid the release of an undesirable metabolic product into a food, or the gene can be replaced with the homologous gene from another strain (Sasaki, 1994; Ito and Sasaki, 1994). For example, if a strain of *Lactococcus* has a particularly useful characteristic, such as the secretion of a desirable flavour component, but the level of β-galactosidase activity is low, then this latter deficiency could be corrected by introducing a more active copy of the gene from another strain (Yu *et al*., 1983; Kochhar *et al*., 1992). Genes could be inserted into a strain to expand the range of carbohydrates utilised (Branny *et al*., 1993, 1996) or increase resistance to a wider spectrum of bacteriophage or, alternatively, a useful gene within the existing genome can be copied, so doubling the beneficial activity (Mollet and Delley, 1991).

An example of the potential offered by these techniques relates to the production of diacetyl, a major flavour component of buttermilk and kefir and a compound that is usually derived via pyruvate. If genes coding for α-acetolactate synthase, an enzyme involved in the conversion of pyruvate to diacetyl, could be inserted into a food-grade culture, diacetyl production would increase and the same approach could be employed in the synthesis of EPS by *S. thermophilus* or *L. delbrueckii* subsp. *bulgaricus*. The relevant genes have been identified from several strains and GMOs with altered texture-producing properties could be constructed (Gasson, 1997).

Exactly how far and fast the construction of GMOs will proceed – or will be allowed to proceed – remains to be seen, but it seems likely that: (a) the identification of species within starter cultures is going to become increasingly imprecise as the borderlines between, for example, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* become blurred as a result of genetic manipulation, and (b) future generations of yoghurt makers will be able to request the supply of starter cultures with quite specific characteristics.

In view of the wide range of technical data available on the genetic modifications of the yoghurt and bio starter cultures, it is recommended that the reader consults

some selected publications for general information (Nicholson and Sanders, 1988; le Bourgeois *et al*., 1989; Schmidt *et al*., 1989; Miteva *et al*., 1991; Yohda *et al*., 1991; Schroeder *et al*., 1991; Leong-Morgenthaler *et al*., 1991; Janzen *et al*., 1992; Pébay *et al*., 1992; Delcour *et al*., 1993; Poolman, 1993; Mustapha *et al*., 1995).

6.2 Characteristics of growth

Yoghurt and the many fermented milks known across the world have been traditionally made by the spontaneous growth of indigenous micro-organisms present in milk. At present, carefully controlled microbial processes have been developed using selected combinations of cultures and the technology required for large-scale production has evolved from the knowledge of the physiology and biochemistry of the micro-organisms involved (refer to Chapter 7).

Since the late 1970s much work has been done on the biochemistry and molecular biology of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Catabolism is not the only important consideration for a successful fermentation to produce yoghurt of good quality in terms of flavour and stability, but anabolic pathways also have a role in providing texture-modifying polysaccharides and providing other compounds which have preservative and health-promoting properties.

6.2.1 Milk as a medium for microbial growth

Lactic acid bacteria are widely distributed in nature and their nutritional requirements are very complex. Table 6.1 shows the fermentation ability and growth temperatures of the yoghurt starter cultures and some of these characteristics are used to differentiate the genera and species. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and many other lactic acid bacteria are unable to synthesise a full complement of amino acids and this deficiency dictates their natural habitat. Milk is a nutritionally rich medium which will support the growth of many micro-organisms, but the processing of milk provides control over the type of growth necessary to achieve a desirable product (see Chapter 2; Chandrakanth *et al*., 1993).

The metabolic activity of an organism is indicative, to some extent, of its growth rate, and one of the most popular tests for monitoring starter cultures is the development of acidity in the growth medium. Autoclaved, reconstituted skimmed milk (10–12 g total solids (TS) 100 g^{-1}) is mainly used and the milk must be free from any inhibitory substances, for instance antibiotics. The activity of a typical yoghurt starter culture and the isolated strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* is illustrated in Fig. 6.1 which shows a marked difference in the rate of acid development by the mixed starter compared with the isolated single strains. It is also noticeable that the rate of acid development of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* increases with increase in incubation temperature, up to maxima of 40°C and 45°C, respectively; the former organism is initially more active than *L. delbrueckii* subsp. *bulgaricus* in relation to acid production. Although the activity of mixed strains is optimum at 45°C, it is recommended that, in order to maintain and/or achieve a ratio of 1:1 between *S. thermophilus* and *L. delbrueckii*

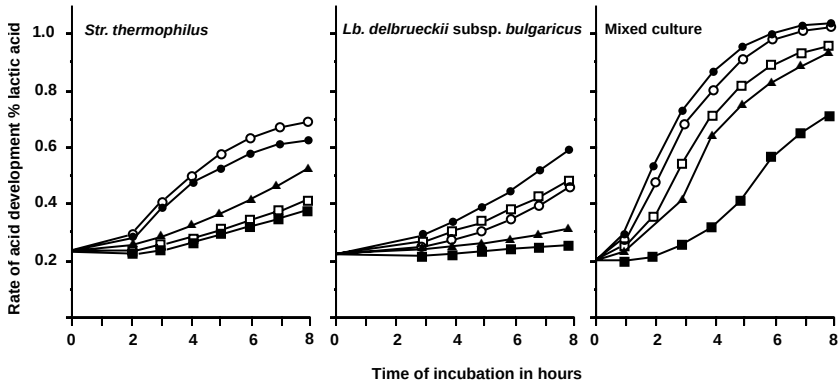

Fig. 6.1 Behaviour of single and mixed strain yoghurt cultures propagated at different temperatures in autoclaved skimmed milk (10 g TS 100 g^{-1}) at 2 ml 100 ml^{-1} inoculation rate ■, 30°C; ▲, 35°C; ○, 40°C; ●, 45°C; □, 50°C.

Note: Test organisms is Chr. Hansen's (CH-1).

Adapted from Tamime (1977a).

subsp. *bulgaricus*, the organisms should be propagated together at 42°C using a 2 ml 100 ml^{-1} inoculation rate (Kurmann, 1967; Tamime, 1977a) or direct-to-vat inoculation (DVI).

6.2.2 Associative growth

The growth association between the two organisms *(S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus)* of the yoghurt starter culture used to be termed a symbiosis and this relationship has been reported by many workers; the earliest record dates back to the work of Orla-Jensen (1931). This association could be briefly described as each organism providing compounds which benefit the other. Since both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* can grow in milk as single cultures, the term symbiosis should be replaced by associative growth instead. Pette and Lolkema (1950a) observed that the rate of acid development was greater when mixed yoghurt cultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were used compared with the single strains (see Fig. 6.2; Lee *et al*., 1990a). Furthermore, they also observed that the numbers of *S. thermophilus*, as recorded by the Breed smear method, were much higher in mixed cultures than when the organism was grown alone, although no such differences in numbers of *L. delbrueckii* subsp. *bulgaricus* were noted. This observation was not true with respect to *L. delbrueckii* subsp. *bulgaricus* as reported by Tamime (1977b). The findings of Pette and Lolkema (1950b) led them to postulate that the interaction between these two organisms was mainly dependent on the production of valine by *L. delbrueckii* subsp. *bulgaricus.* However, due to variations in the chemical composition of milk during the year, other amino acids may also be deficient and hence Pette and Lolkema (1950c) suggested that during the spring months, *S. thermophilus* required amino acids leucine,

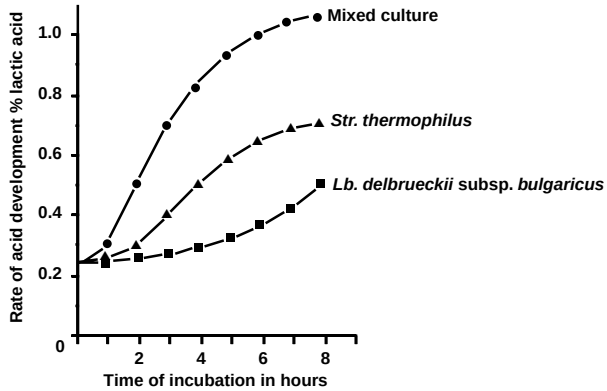

Fig. 6.2 Behaviour of single and mixed strain yoghurt cultures propagated at 40°C in autoclaved skimmed milk (10 g TS 100 g^{-1}) at 2 ml 100 ml^{-1} inoculation rate

Note: Test organism is Chr. Hansen's (CH-1).

Adapted from Tamime (1977a).

lysine, cystine, aspartic acid, histidine and valine. During the autumn/winter months, glycine, isoleucine, tyrosine, glutamic acid, methionine, as well as the six amino acid mentioned above, were essential.

Bautista *et al.* (1966) also investigated the associative growth theory and supported the view that *L. delbrueckii* subsp. *bulgaricus* stimulates *S. thermophilus* by releasing glycine and histidine into the growth medium; they concluded that histidine rather than valine was the most important requirement. However, the stimulation by glycine and histidine, as reported by Bautista *et al.* (1966), was very poor in comparison with the various amino acids observed by Pette and Lolkema (1950b). Accolas *et al.* (1971) reported that the stimulation of *S. thermophilus* by milk culture filtrate of *L. delbrueckii* subsp. *bulgaricus* was due to the presence of valine, leucine, isoleucine and histidine. Bracquart *et al.* (1978) and Bracquart and Lorient (1979) concluded that depleting the growth medium of valine, histidine, glutamic acid, tryptophan, leucine and isoleucine reduced the stimulation of *S. thermophilus* by 50%. Similar findings were reported by Higashio *et al.* (1977a), where methionine was also included as a stimulant amino acid; however, by far the most effective amino acid was valine (see also Shankar, 1977; Shankar and Davies, 1978; Hemme *et al.*, 1981; Rao *et al.*, 1982; Marshall, 1983).

It is well established that *L. delbrueckii* subsp. *bulgaricus* possesses more proteolytic enzymes than *S. thermophilus* (see Chapter 7; Rajagopal and Sandine, 1990; Abu-Tarboush, 1996) and El-Soda *et al.* (1986) reported that crude cell-free extracts of the yoghurt lactobacilli stimulated the growth of *S. thermophilus*; they concluded that acid production was enhanced by the addition of peptone, amino acids and, to a lesser extent, water-soluble vitamins, purines and pyridines. A similar view was reported by El-Abbassy and Sitohy (1993) and Neviani *et al.* (1995), whilst Carmi-

nati *et al*. (1994) concluded that a skimmed milk medium deprived of soluble nitrogen inhibited the growth of *S. thermophilus*. Other amino acids, which are not the result of proteolysis by the yoghurt organisms, that have stimulated the growth of *S. thermophilus* are: (a) peptides containing lysine (Desmaseaud and Hermier, 1972), (b) hepta- or pentapeptides containing histidine and free non-aromatic amino acids (Desmaseaud and Hermier, 1973; Hayashi *et al*., 1974), (c) tripeptides containing histidine, methionine and glutanic acid (Bracquart and Lorient, 1979), (d) casein hydrolysate (Marshall and Mabbitt, 1980; Marshall *et al*., 1982; Nakamura *et al*., 1991), and (e) the addition of magnesium (Amouzou *et al*., 1985). However, the transport of branched amino acids in *S. thermophilus* is energy dependent and optimum activity was between 30°C and 45°C for leucine, valine and isoleucine (Akpemado and Bracquart, 1983). Other technical data available on the associative growth of the yoghurt organisms have been reported by Radke-Mitchell and Sandine (1984), Matalon and Sandine (1986), Juillard *et al*. (1987), Berkman *et al*. (1990), Kneifel *et al*. (1993) and Oberg and Broadbent (1993) (see also Champagne *et al*., 1990; Klaver *et al*., 1992; Franzetti *et al*., 1997).

Thus, the streptococci benefit from the stronger activity of the lactobacilli and in return provide certain compounds which stimulate the growth of *L. delbrueckii* subsp. *bulgaricus*. However, glutamic acid uptake in *S. thermophilus* was energy dependent (e.g. lactose, glucose and sucrose), but aspartic acid exhibited an inhibitory effect (Benateya *et al*., 1986; Bracquart *et al*., 1989).

Galesloot *et al.* (1968) investigated the opposite side of the associative growth relationship between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. They concluded that, under anaerobic conditions, the former organism produces a stimulatory factor for *L. delbrueckii* subsp. *bulgaricus* that is equal to or can be replaced by formic acid. Furthermore, the same workers looked at the effect of various heat treatments on milk, and found that in intensively heated milk (i.e. autoclaved and UHT) the stimulation was masked on account of a compound which could be replaced by formic acid. However, after the normal heat treatment of milk used for yoghurt manufacture (e.g. 85–90°C), *L. delbrueckii* subsp. *bulgaricus* definitely needs the stimulatory factor produced by *S. thermophilus*. The normal presence of this stimulatory factor in autoclaved milk (Auclair and Portman, 1957; Shankar, 1977; Marshall, 1983), appears to have been overlooked by both Pette and Lolkema (1950b) and Bautista *et al*. (1966).

The production of formic acid by *S. thermophilus* was confirmed by Veringa *et al.* (1968), and Bottazzi *et al.* (1971) demonstrated that the presence of formic acid in milk increases the ratio of rods to cocci at concentrations between 30 and 50 $\gamma\,ml^{-1}$. This compares with the stimulation of *L. delbrueckii* subsp. *bulgaricus* by formate at 20–30 $\mu g\,ml^{-1}$ (Galesloot *et al*., 1968; Shankar, 1977; Marshall, 1983) and 40–600 $\mu g\,ml^{-1}$ (Accolas *et al*., 1971; Pulsani and Rao, 1984; Kikuchi *et al*., 1985; El-Abbassy and Sitohy, 1993; Moreira *et al*., 1997). This variation in the level of formate required to promote activity could be attributed to the use of different strains of *L. delbrueckii* subsp. *bulgaricus*. Also, the amount of formate production by *S. thermophilus* is dependent on strain, culture medium and growth temperature (Perez *et al*., 1990, 1991); the streptococci produce formic acid in milk only if the level of oxygen $\leq 4\,mg\,O_2\,l^{-1}$ (Driessen *et al*., 1983).

Some *L. delbrueckii* subsp. *bulgaricus* strains grown in milk heated to 100°C for 15 min showed an abnormal cell elongation, and septum staining indicated that the septum had not yet formed. However, such morphological behaviour was not

observed in autoclaved milk and/or milk heated to 100°C for 15 min (Suzuki *et al*., 1986). Furthermore, the presence of sodium formate (40 μg ml^{-1}) in milk induced the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* so that it became able to hydrolyse β-Lg, α_{s1}- and β-casein compared to only β-casein without the added formate (Moreira *et al*., 1997).

Carbon dioxide, which is produced by *S. thermophilus* (Ascon-Reyes *et al*., 1995) had been reported by Driessen *et al*. (1982) to stimulate the growth of *L. delbrueckii* subsp. *bulgaricus* because part of the CO_2 produced by the streptococci disappears during mixed growth with the lactobacilli. CO_2 is produced as a result of urea hydrolysis and can be measured using an indirect conductance technique (Ascon-Reyes *et al*., 1995; see also Lanzanova *et al*., 1993), whilst measuring partial pressure of dissolved CO_2, the concentration of viable cells of the yoghurt micro-organisms could also be determined (Spinnler *et al*., 1987). CO_2 production in dahi incubated at 42°C using 1 ml 100 ml^{-1} starter culture amounted to about 450 μl, and Warsy (1983) suggested that the gas produced may contribute to the sensory quality of the product. However, in a recent study, Louaileche *et al*. (1993, 1996) reported that CO_2 and sodium bicarbonate stimulated the growth of *S. thermophilus*, and exerted a marked influence on the metabolic activities of the micro-organism, a phenomenon that has not been reported before.

Other compounds produced by *S. thermophilus* that stimulate the growth of *L. delbrueckii* subsp. *bulgaricus* are pyruvate and HCO_3 (Higashio *et al*., 1977b, 1978; Juillard *et al*., 1987). Other added compounds that stimulated the growth of the lactobacilli are purine, adenine, guanine, uracil and adenosine (Weinmann *et al*., 1964; Cogan *et al*., 1968), monosodium orthophosphate and sodium tripolyphosphate (Yu and Kim, 1979), oxaloacetic and fumaric acid (Higashio *et al*., 1977b) and cysteine at ≤ 50 mg l^{-1} (Dave and Shah, 1997). Nevertheless, the action of psychrotrophic bacteria in milk, fortification of the solids of the milk base and/or heating of the milk can also promote the growth of the yoghurt starter culture (Tramer, 1973; Cousins and Marth, 1977a, b; Sellars and Babel, 1985; Slocum *et al*., 1988a, b; for further information refer to Chapter 2).

It can be concluded from the data available, therefore, that the release of stimulatory factors by the yoghurt starter cultures takes place during the incubation period and, while *L. delbrueckii* subsp. *bulgaricus* provides essential nutrients (i.e. amino acids) for *S. thermophilus*, the latter produces formate which promotes the growth of the lactobacilli. Alternatively, the growth characteristics of the yoghurt organisms can be increased through the application of an electromagnetic field (Blicq and Murray, 1994) or the use of surface methodology to evaluate some variables affecting the growth behaviour of the yoghurt organisms (Torriani *et al*., 1996).

Amoroso and Manca de Nadra (1990) observed the mutual stimulation in milk, while in LAPT medium (containing yeast extract, peptone, tryptone and Tween) with different sugars, only the stimulatory effects of the *Streptococcus* on the *Lactobacillus* were observed. This is an expected result as the nitrogen sources in LAPT medium are readily available and not dependent on proteolytic activity (the mechanism for stimulation of the *Streptococcus* is the release of peptides by the lactobacilli); thus, the medium used could demonstrate only one side of the partnership. This underlines the importance of understanding the special qualities of milk as a growth medium; it has an ample supply of a simple disaccharide and an ample, but complex source of nitrogen. It is also important to remember that both organisms

grow perfectly well in milk. Indeed, many of the mild bio-yoghurts are prepared with mixed cultures, some of which include *L. delbrueckii* subsp. *bulgaricus* for a successful fermentation (see also Marshall and Tamime, 1997).

Although in the 1980s, *S. thermophilus* was temporarily included as a subspecies of *Streptococcus salivarius* (Farrow and Collins, 1984), a separate species was proposed by Schleifer *et al.* (1991); *S. salivarius* fails to grow in milk in the presence of *L. delbrueckii* subsp. *bulgaricus* and is not suitable for the manufacture of yoghurt because of poor flavour, aroma and texture (Marshall *et al.*, 1985). Such observations may also justify the revival of the species, *S. thermophilus*, even though both species have similar DNA base compositions and belong in the same DNA homology group. Nevertheless, associative growth was reported between *S. thermophilus* and *L. helveticus* or *L. acidophilus* (Yoon *et al.*, 1988; Kim *et al.*, 1992) and bifidobacteria stimulated the growth of yoghurt starter cultures (Kumar *et al.*, 1995).

6.3 Factors affecting slow growth of starter cultures

Yoghurt microflora can easily grow in milk and the rate of acid development is faster due to the growth associated with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (see Fig. 6.2 and Section 6.2). Nevertheless, the fermentation conditions and the presence of certain agents or substances in milk may either reduce the rate of acid development or inhibit growth of the culture and these aspects are summarised in the following section.

6.3.1 Compounds that are naturally present in milk

There are various antimicrobial systems present in milk and their major role is the protection of the suckling animal against infection and disease. These inhibitory systems have been reviewed by Reiter (1978) and their presence in milk can inhibit the growth of lactic acid bacteria. Auclair and Hirsch (1953) and Auclair and Berridge (1953) reported the inhibition of starter organisms by raw milk and that pasteurisation and boiling of the milk improved culture activity. The inhibitory compounds, known as lactenins, are heat sensitive, and are destroyed by heating the milk to 68–74°C (Auclair, 1954). Patel (1969) reported that *S. thermophilus* showed a growth inhibition in fresh raw buffalo's milk during the first 1–2 hour of incubation, but a resumptation of growth followed. He proposed that the loss of inhibitory action was due either to adaptation of the organism to the lactenins or to the destruction of the lactenins.

Another bactericidal component found naturally in milk is the peroxidase system, which consists of lactoperoxidase/thiocyanate/hydrogen peroxide [LP/SCN^-/H_2O_2 abbreviated as LPS]. Reiter (1978) reported on the sources of these compounds.

- LP is synthesised in the mammary gland and milk may contain up to 30 $\mu g\,ml^{-1}$ peroxidase which is sufficient to activate the LPS (Reiter, 1985; Nichol *et al.*, 1995).
- SCN^- anion is widely distributed in animal secretions and possibly derived from a rhodanese catalysed reaction with thiosulphate in the liver and kidney; the SCN^- concentration in milk may reach up to 10–15 $\mu g\,g^{-1}$ (Reiter and

Härnulv, 1984; Reiter, 1985; Haddadin *et al.*, 1996; see also Prasad and Sukumaran, 1992).

- H_2O_2 does not occur naturally in milk (Piard and Desmazeaud, 1991; Nichol *et al.*, 1995), but its presence in milk is the result of metabolic activity of the lactic bacteria or from anaerobic growth of other micro-organisms.

In this system, the inhibitory compound is the result of an oxidation reaction where, in the presence of H_2O_2, the LP catalyses the oxidation of thiocynate to non-inhibitory compounds (SO_4^{2-}, CO_2 and NH_3) followed by further oxidation to form intermediate inhibitory substances, such as hypothiocyanate or higher oxyacids (Piard and Desmazeaud, 1991; Björck, 1992; Dionysius *et al.*, 1992; Grieve *et al.*, 1992). However, the inhibition is reversible in the presence of some reducing compounds (e.g. cysteine and dithionite; Reiter, 1978). In general, most starter organisms are resistant to LP systems, but some lactic cultures can give rise to sensitive mutants (Auclair and Vassal, 1963). Alternatively, continual propagation of starter cultures in autoclaved milk can affect the susceptibility of the organisms to the LP system (Jago and Swinbourne, 1959). A preventive measure is the addition of peroxidase to autoclaved milk (Reiter, 1973), or the addition of reducing agents, like cysteine and dithionite (Reiter, 1978). Incidentally, the LP system is inactivated by heating milk at 85°C for 16 s (Feagan, 1959a, b), so that heat treatment of yoghurt milk (85°C for 30 min or 90–95°C for 5–10 min) and the bulk starter milk (93°C for $1\frac{1}{2}$–2 hours) are sufficient to destroy the natural inhibitors (Storgards, 1964; Pearce and Bryce, 1973; Ekstrand *et al.*, 1985; Farkye, 1992). Thus, since H_2O_2 does not occur naturally in milk, the mechanism(s) involved in production and inhibition of the yoghurt organisms is given in detail in Section 6.3.4.

Other inhibitory systems which may warrant some consideration are: (a) bacterial agglutinin which can cause agglutination of the starter organisms, thus affecting their metabolic activity and growth, and (b) certain types of forage, such as mouldy silage, turnips or vetch, which may result in a milk containing inhibitory substances which can reduce the rate of acid production of the yoghurt starter culture, even after heating the milk at 90°C for 15 min (see the review by Tamime and Deeth, 1980).

6.3.2 Effect of incubation temperature and inoculation rate

The growth behaviour of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (i.e. as single and/or mixed cultures) has been shown in Fig. 6.1 and it is evident that when the starter culture is incubated at 40–50°C, the optimum rate of acid development is obtained within a very short period. However, in industrial situations, yoghurt is produced over a short or long period using incubation temperatures at 30°C or 45°C, respectively. In the former method of production, a reduced rate of acid development becomes inevitable and, although this effect is governed by processing conditions, the quality of the end product could be affected. Some published data are available and it is recommended that the reader consult the following publications for general information (Tayeb *et al.*, 1984; Mohanan *et al.*, 1984; Radke-Mitchell and Sandine, 1986; Jayaram and Gandhi, 1987; Cho-Ah-Ying *et al.*, 1990; Béal and Corrieu, 1991; Lankes *et al.*, 1998).

The inoculation rate can also affect the rate of acid develoment during the manufacture of yoghurt. For example, an additional rate of 2–3 ml 100 ml^{-1} bulk

starter culture is recommended, whilst a DVI inoculation rate may range between 2.5 and 70 g $100l^{-1}$ depending on the starter culture blend used (i.e. standard or bio culture). Thus, an inaccurate rate of starter addition to the milk base can affect the rate of acid development of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.

6.3.3 Mastitis milk and somatic cell count

Gajdusek and Seleba (1973) reported a 35% reduction in the activity of a yoghurt culture in milk containing large numbers of somatic cells; however, boiling the milk for 2 min, or heating to 90°C for 20 min, inactivates the cells completely. Whilst somatic counts of 4.0×10^5 cells ml^{-1} cause some inhibition of growth of the yoghurt organisms with *S. thermophilus* less resistant than *L. delbrueckii* subsp. *bulgaricus*, complete inhibition of both organisms occurs at counts $>1.0 \times 10^6$ cell ml^{-1} (Mitic *et al.*, 1982). However, Marshall and Bramley (1984) and Okella-Uma and Marshall (1986) reported stimulation of *S. thermophilus*, but inhibition of *L. acidophilus*, when these organisms were grown in mastitic milk containing high somatic cell counts. The stimulation was attributed to increased proteolysis and the inhibition to increased phagocytic activity of the polymorphonuclear leukocytes. However, Fang *et al.* (1993) observed only reduced growth activity of *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus paracasei* subsp. *paracasei* in mastitic milk. These reported differences in the growth behaviour of *L. acidophilus* could be strain related.

The quality of yoghurt made from skimmed milk containing a somatic count of $\leq 2.5 \times 10^5$ cells ml^{-1} was organoleptically superior to a parallel product made from milk of $\geq 2.5 \times 10^5$ cells ml^{-1} (Mitchell *et al.*, 1985; Rogers and Mitchell, 1994). Thus, from the limited data available in this field, it is recommended that yoghurt producers should use milk with a low somatic cell count as reported by Rogers and Mitchell (1994) (see also Auldist and Hubble, 1998).

6.3.4 Hydrogen perxoide (H_2O_2)

Hydrogen peroxide is added to raw milk produced in hot countries to improve its quality during storage. The recommended rate to activate LPS system is 3 mg $100 g^{-1}$ of sodium percarbonate ($2Na_2CO_3 \times 3H_2O_2$) and 1.4 mg $100 g^{-1}$ of sodium thiocyanate (NaSCN) (IDF, 1988b). However, the natural presence of H_2O_2 in milk and activation of LPS, which can inhibit the growth of lactic acid bacteria and other micro-organisms, is the result of sugar metabolism during fermentation. A wide range of reactions and catalysing enzymes are involved and these have been recently reviewed by Condon (1987) and Piard and Desmazeaud (1992).

Oxygen uptake activity and aerobic metabolism of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have been reported by Smart and Thomas (1987), Teraguchi (1987), Teraguchi *et al.* (1987) and Condon (1987). H_2O_2 produced by the *Lactobacillus* in the presence of glucose at pH values of 6.5 and 5.0 was apparently due to the action of cytosolic NADH oxidase (Kot *et al.*, 1996, 1997). Schuts *et al.* (1982) reported that the amount of H_2O_2 (0.8 to 1.8 mg $100 ml^{-1}$) produced by *L. delbrueckii* subsp. *bulgaricus* was influenced by the strain, growth medium and the type of added sugars; the highest amount of H_2O_2 was obtained in UHT milk. However, lactic acid bacteria can rid themselves of H_2O_2 formed only by their NADH peroxidase (Piard and Desmazeaud, 1991). The ability of the yoghurt organisms to

consume oxygen in milk was about 0.4 mg 100 ml^{-1} in 24 hour at 25°C (Langeveld and Bolle, 1985), whilst the influence of dissolved O_2 on acid production in buffalo's milk by *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and lactococcal species has been studied by Shekar and Bhat (1983). However, *L. acidophilus*, *S. thermophilus* and some bifidobacterial strains, but not *L. delbrueckii* subsp. *bulgaricus*, could transport Fe^{2+} into the cell where it is partially oxidised to the ferric form (Kot *et al*., 1995); *L. delbrueckii* subsp. *bulgaricus* could only oxidise extracellular Fe^{2+} through the elaboration of H_2O_2 in the presence of glucose and air.

Therefore, the LPS system can be activated in the presence of H_2O_2 via two possible routes, the first due to the metabolic activity of the starter cultures and the second, by the addition of thiocyanate and H_2O_2. Zall *et al*. (1983) reported that when the latter approach was used with rates of 0.2 mM and 0.25 mM, respectively, it extended the shelf life of raw milk up to 8 days without substantially increasing the total viable count, but when such milk was used for the manufacture of buttermilk, Cheddar cheese or yoghurt, culture activity was reduced. Nichol *et al*. (1995) reported self-induced inhibition of *S. thermophilus* by activation of LPS, whilst activation of LPS system by adding H_2O_2 and thiocyanate suppressed acid production during the manufacturing stages and refrigerated storage of yoghurt (Mehanna and Hefnawy, 1988; Kumar and Mathur, 1989; Basaga and Dik, 1994; Sarkar and Misra, 1994; Nakada *et al.*, 1996). In a simulated system, *L. acidophilus* (one strain) and *L. delbrueckii* subsp. *bulgaricus* (three strains) were inhibited in the presence of lactoperoxidase and thiocyanate indicating their ability to produce H_2O_2 to complete the LPS system, whilst *S. thermophilus*, *L. helveticus* and *Lac. lactis* subsp. *lactis* (one strain) required an external source of H_2O_2 to cause inhibition by the LPS system (Guirguis and Hickey, 1987a). The same authors also reported that one strain each of *L. delbrueckii* subsp. *bulgaricus*, *L. lactis* subsp. *lactis* and *Enterococcus faecium* were resistant to LPS system.

It is evident that the LPS system may inhibit or act as a bacteriostatic agent of the yoghurt starter cultures. Such effects may possibly depend on the rate of accumulation and/or reduction of the H_2O_2 (i.e. the activities of NADH oxidase and NADH peroxidase) in the bacterial cell. Therefore, screening of the yoghurt organisms in relation to the effect of the LPS system may help to overcome production problems at certain period(s) of the year, stages of lactation, or thiocynate and H_2O_2 must be used at lower levels than recommended by IDF (1988b).

6.3.5 Antibiotic residues

Antibiotics and/or other antimicrobial agents are used for the treatment of diseases. One of the major diseases in the dairy cow, which can affect the quality and yield of milk, is mastitis. Today there are known to be about 1000 different types of antibiotic and the following antimicrobial compounds (penicillin, streptomycin, neomycin, chloramphenicol, tetracycline, sulphonamide, cloxacillin and ampicillin) are widely used in the United Kingdom for the treatment of mastitis. The presence of these antibiotics in milk can either inhibit the growth or reduce the activity of the yoghurt starter cultures. The sensitivity of these organisms (i.e. single strains or mixed culture) to these various compounds is shown in Table 6.3 (see also Park *et al.*, 1984; Sinha, 1984; Hsu *et al*., 1987; IDF, 1987, 1991a; Herian *et al*., 1990; Milashki, 1990; Schiffmann *et al*., 1992; Celik, 1992; Brindani *et al*., 1994).

Table 6.3 Sensitivity of the yoghurt starter cultures to various antibiotics (ml^{-1})

Antibiotics	Micro-organisms		
	S. thermophilus	*L. delbrueckii* subsp. *bulgaricus*	Mixed culture (IU)
Penicillin	0.004–0.01 IU	0.02–0.1 IU	0.01
Streptomycin	0.38 IU	0.38 IU	1.0
	12.5–21.0 µg	6.6 µg	NR
Tetracycline	0.13–0.5 µg	0.3–2.0 µg	1.0
Chlortetracycline	0.06–1.0 µg	0.06–1.0 µg	0.1
Oxytetracycline	0.4 IU	0.7 IU	0.4
Bacitracin	0.04–0.12 IU	0.04–0.1 IU	0.04
Erythromycin	0.3–1.3 mg	0.7–1.3 mg	0.1
Chloramphenicol	0.8–13.0 mg	0.8–13.0 mg	0.5

IU, international units; NR, not reported.

Data compiled from Tamime and Deeth (1980), Loussouarn (1983), Schiffmann (1993) and Lim *et al.* (1995).

During the intramammary injection of antibiotics for the treatment of mastitis in the dairy cow, these antimicrobial compounds are retained in the udder tissues and gradually diffuse into the milk.

Thus, milk from treated cows must be withheld for 72 hours for two main reasons. First, residual antibiotics in milk are a potential public health hazard and second, low levels can affect the behaviour and activity of the starter culture (see Table 6.3), resulting in a poor yoghurt and/or economic loss for the manufacturer. Hence, a number of governments have introduced a payment penalty scheme for milk containing >0.004 International Units (IU) of penicillin ml^{-1}; among the test methods are the disc assay, the 2,3,5-triphenyltetrazolium chloride (TTC), bromocresol purple (BCP) or the Charm test (see IDF, 1991a and Chapter 10). Some of these methods use *S. thermophilus* as the test organism because of its sensitivity to antibiotics (see Table 6.3), but unfortunately the available methods are prone to certain drawbacks:

- The sensitivity of *S. thermophilus* can vary in relation to the strain used (see Reinbold and Reddy, 1974).
- The above test methods may have certain limitations, for example, Cogan (1972) observed that *L. delbrueckii* subsp. *bulgaricus* is more sensitive than *S. thermophilus* to streptomycin, and to cause a 50% inhibition of growth, 1.6–4.45 and 7.3–13.00 µg ml^{-1} of streptomycin were required, respectively. Thus, a milk which passes the antibiotic test may contain enough streptomycin to inhibit the growth of *L. delbrueckii* subsp. *bulgaricus* (see also Park *et al*., 1984). However, comparative growth of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in milk containing streptomycin showed that the latter micro-organism was more sensitive (Ramakrishna *et al*. (1985); again strain differences appear to be important.

The major effect of antibiotic residues in yoghurt milk is to cause a breakdown in the associative growth between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus,* or a slow down in the rate of acid development (i.e. longer processing time)

and this can, in turn, lead to syneresis or wheying-off. To combat such problems, the following measures have been recommended:

- The use of milk for the manufacture of yoghurt that is free from antibiotics.
- The addition of penicillinase or penicillinase-producing organisms, e.g. *Micrococcus* spp., to milk in order to inactivate residual penicillin contamination (Reiter *et al*., 1961; Vazquez and Reiter, 1962).
- Heat treatment of milk can reduce the potency of some antibiotics. Tramer (1973) reported an 8% inactivation of penicillin at 72°C for 15 s, or 20% at 87.7°C for 30 min, or 50% at commercial sterilisation temperatures; tetramycin lost 2/3 of its potency at 85°C for 30 min, but streptomycin and chloramphenicol remained stable and unaffected.
- Lowering the water activity of the growth medium with glycerol for *S. thermophilus* (A_w from 0.992 to 0.995) and *L. delbrueckii* subsp. *bulgaricus* (A_w from 0.992 to 0.985) improved the resistance of these organisms against penicillin, but not gentamycin (Larsen and Anon, 1989b).

It is most likely that the inhibitory effect on these organisms is influenced by the mode of action of the antibiotics and, in view of the immense number of antimicrobial drugs used in veterinary medicine, an attempt has been made to classify only the most widely used antibiotics. The overall characteristics of this group and their possible effect on the yoghurt starter cultures is shown in Table 6.4. Furthermore, depending on the type of antibiotic used, the mode of action of these drugs on *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* can be summarised as follows: (a) interference with the cell membrane structure and permeability, (b) interference with cellular metabolism of proteins, carbohydrates and lipids, (c) interference with energy-yielding transformations in the cell, (d) inhibition of various enzymes and phosphorylation systems, and (e) blocking the synthesis of DNA and RNA during cell division.

Antibiotic-resistant yoghurt strains (see Table 6.5) have been induced to resist higher concentrations of antibiotics by repeated subculturing in milk containing varying concentrations of the antibiotics (Babu *et al*., 1989a; see also Yondem *et al*., 1989; Bozoglu *et al*., 1996). However, the quality of yoghurt produced by such strains was not reported, but Babu *et al*. (1989a) reported the penicillin-resistant *L. delbrueckii* subsp. *bulgaricus* showed almost 50% reduction in acetaldehyde production, whilst the streptomycin-resistant cultures exhibited appreciable depression in flavour production. Thus, these developed cultures may have different characteristics, such as reduced rates of acid and flavour production, or the inability to ferment certain carbohydrates, and these changes could adversely affect the performance of a culture during commercial production (see Babu *et al*., 1989a, b; Chirica *et al*., 1998). Furthermore, genes for drug-resistance play an important role as genetic markers, and spontaneous frequencies of mutation to antibiotic resistance interfere with genetic research for the improvement of starter cultures for fermentation (Curragh and Collins, 1992).

6.3.6 Detergent and disinfectant residues

Detergents and disinfectants are widely used in the dairy industry for cleaning and sanitising dairy equipment on the farm and in the creamery (see Chapter 4). The general specification and classification of these preparations is discussed elsewhere,

Table 6.4 Classification and mode of action of some antibiotics

Source or origin	Antibiotics produced	Production (%)	Possible function and mode of action on the yoghurt starter culture
Microbial			
Streptomyces spp.	Streptomycin	58	* Protein synthesis inhibitors
	Tetracyclines		** Protein synthesis inhibitors
	Neomycin		* Protein synthesis inhibitors
	Erythromycin		* Protein synthesis inhibitors
	Chloramphenicol		** Protein synthesis inhibitors
Nocardia spp.	Ristocetin		* Cell wall inhibitors
*Micromonospora s*pp.	Gentamicin		* Protein synthesis inhibitor
Penicillin notatum	Penicillin Xanthocillin	18	* Cell wall inhibitors
Fusidium coccineum	Fusidic acid		Nucleic acid inhibitors
Aspergillus fumigatus	Fumagillin		
Bacillus licheniformis	Bacitracin	9	* Cell wall inhibitors
Bacillus brevis	Gramicidins		* Alter cell membrane permeability
	Tyrocidin		* Disorganise cell membrane structure
Bacillus polymyxa	Polymyxin		* Disorganise cell membrane structure
Synthetic	Sulphonamide	12	Reaction or site inhibited is folate synthesis
	Penicillin		* Cell wall inhibitors
	Chloramphenicol		** Protein synthesis inhibitors
Plant extracts	Alkaloids		
Miscellaneous	Drugs extracted from algae, lichens and animals	3	

* Bactericidal. ** Bacteriostatic.

Adapted from Garrod *et al*. (1973) and Edwards (1980).

but basically, the detergent formulations contain alkali compounds (e.g. sodium hydroxide), while the sanitising agents are quaternary ammonium compounds (QAC) or iodine or chlorine-based compounds.

Inorganic acids are also used for cleaning and disinfecting purposes. Therefore, residues of these compounds in milk can be attributed to two main causes. First negligence, bad management or a faulty cleaning-in-place (CIP) system (i.e. on the farm or at the factory); the latter is more likely to occur on the farm or in milk tankers.

Table 6.5 Development of yoghurt starter cultures resistant to different antibiotics

Antibiotics	Achieved resistance (ml^{-1})	References
Penicillin Streptomycin	3 IU 500 µg	Hargrove *et al.* (1950)
Chlortetracycline Chloramphenicol Streptomycin	70–120 µg 40–50 µg 500 µg	Solomon *et al.* (1966)
Ampicillin Cephalexin Chlortetracycline	50 µg 150 µg ≤50–150 µg	Ferri *et al.* (1979)
Penicillin Streptomycin	0.25 IU 500 µg	Babu *et al.* (1989a, b)

Table 6.6 Sensitivity of the yoghurt starter cultures to various detergent disinfectants and pesticides ($mg\ l^{-1}$)

Inhibitory substances	Micro-organisms		
	S. thermophilus	*L. delbrueckii* subsp. *bulgaricus*	Mixed culture
Disinfectant/detergent			
Chlorine compounds	5–100	2.5–100	50–>2500
QAC	100–500	0.5–100	>250
Ampholyte			>1000
Iodophore	10–60	60	>2000
Alkaline detergent			500–1000
Insecticides			
Malathion			200
N-methylcarbanate			20

Adapted from Tamime and Deeth (1980), Guirguis and Hickey (1987b) and Petrova (1990).

Second, it is the practice of some milk producers overseas to add biocidal compounds (e.g. H_2O_2) to milk in order to improve its keeping quality. This latter approach is not recommended for public health reasons and the presence of such compounds in milk can adversely affect, or totally inhibit, the growth of starter cultures.

It can be observed from Table 6.6 that the susceptibility of *S. thermophilus* and *L. debrueckii* subsp. *bulgaricus* to cleaning residues is increased in monocultures compared with mixed cultures and this variation could be attributed to:

- differences or variations in the strains of bacteria being used by different researchers (Liewen and Marth, 1984; Guirguis and Hickey, 1987b; El-Zayat, 1987; Mäkelä *et al*., 1991);
- variation between batches of the commercial detergents and disinfectants tested;
- variation in the test method used to measure the levels of inhibition (see Lanzanova *et al*. (1991) for the use of a conductimetry technique to evaluate the effects of disinfectants and detergents on the activity of starter cultures);
- greater resistance as a result of associative growth relationships.

Another possible source of detergent and/or sterilant residues is the glass bottle washer, for in some countries, glass bottles are still used for packaging stirred or set yoghurt. In the latter type of yoghurt, Nikolov (1975) concluded that if the milk contained above 2.5% of bottlewash liquid, consisting of 1% sodium hydroxide and hypochlorite (i.e. the chlorine concentration >100 mg l^{-1}), the concentration was high enough to inhibit the growth of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.

6.3.7 Environmental pollution

Incidents of insecticide residues in milk have been reported and this occurrence could well be due either to post-milking contamination, or to feeding cattle with fodder that has been sprayed with an insecticide to combat disease. Milk containing malathion (200 mg l^{-1}) or *N*-methylcarbamate (20 mg l^{-1}) will inhibit the growth of the yoghurt organisms (see Table 6.6). However, Deane and van Patten (1971) observed that 100 mg l^{-1} of malathion or trichlorphon in milk had little effect on the rate of lactic acid development by yoghurt cultures, but some variation in cell morphology did occur after several culture transfers. When viewed under a light microscope (using ordinary staining techniques) the recorded changes included a decrease or increase in cell size and the formation of longer chains. In addition, Deane and Jenkins (1971) propagated *L. delbrueckii* subsp. *bulgaricus* alone in milk containing the same insecticides and observed various morphological changes under the electron microscope. The rod cells were longer, wider or narrower and showed a compact protoplasm and frequent flaking of the cell wall material, and there were fewer cross-walls produced.

In the 1980s, Egyptian scientists intensified their research into the fate of different pesticides (e.g. aldicarb, chlorpyrifos, deltamethrin, lindane, fenvalerate (pyrethroid), malathion and DDT) during the manufacture of zabadi and cheese, and on the growth behaviour of starter cultures (Shaker *et al*., 1985, 1988; Ismail *et al*., 1987; Magdoub *et al*., 1989; Zidan *et al*., 1990; see also Misra *et al*., 1996). The results of these studies could be summarised as follows:

- The pesticide concentration decreased in freshly made zabadi.
- Gelation time of the milk increased and the cheeses had many holes.
- Cells of *L. delbrueckii* subsp. *bulgaricus* floculated into clumps in milk containing aldicarb and the cell count was lower than the control.
- Heating of the pesticide-contaminated milk and fermentation contributed towards the degradation of pesticides.
- Reduced growth rates of *S. thermophilus* in the presence of fenvalerate or DDT were observed, whilst *L. delbrueckii* subsp. *bulgaricus* was sensitive to malathion and DDT.

6.3.8 Bacteriophages

Bacteriophages (phages) are viruses which can attack and destroy the yoghurt organisms and the resultant failure of lactic acid production leads to poor coagulation of the process milk. The occurrence of such viruses in mesophilic dairy starter cultures (e.g. cheese starters) was first reported by Whitehead and Cox (1935) and, for the past few decades, research work on the phages of mesophilic lactic acid

bacteria has been intensified, primarily because of the economic importance of cheese in the dairy industry. However, interest in bacteriophages that can attack thermophilic lactic acid bacteria (i.e. the yoghurt cultures) has been aroused first because world production figures of yoghurt have increased significantly and product failure results in great economic loss to the industry; second because the manufacture of yoghurt is more centralised and bacteriophage attack could become a major problem; and third because strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are widely used in the manufacture of high temperature scalded cheese (e.g. the Swiss varieties) and hence bacteriophage problems could result in both a slow "make" and a low quality cheese. As a consequence, research work on bacteriophages has intensified and a large number of publications are available. However, some selected reviews on bacteriophages of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are recommended for further information (Reinbold and Reddy, 1973; Sozzi *et al*., 1981; Stadhouders *et al*., 1984; Thunell and Sandine, 1985; Ackermann and DuBow, 1987; Mata and Ritzenthaler, 1988; Sechaud *et al*., 1988; Rajagopal and Sandine, 1989; Jarvis, 1989; Cogan and Accolas, 1990; Coffey *et al*., 1994; Sable and Lortal, 1995; Gasson, 1996; Neve, 1996; Auvray *et al*., 1997; Josephsen and Neve, 1998).

The general morphology of a bacteriophage consists of a head and protruding tail, and the type capable of infecting lactic acid bacteria may consist of a double strand of DNA in a linear form which is located in the head (Lawrence *et al*., 1976; Sandine, 1979; Neve, 1996). The guanine plus cystine (G + C) content of the bacteriophage is somewhat similar to the G + C composition of the bacterial hosts' chromosomes; thus in principle, such similarity may explain the close relationship between the bacteriophage and the host. Over the years different methods have been proposed to classify bacteriophages (Pette and Kooy, 1952; Bradley, 1967; Lawrence *et al*., 1976; Soldal and Langsrud, 1978; Koroleva *et al*., 1978; Mullan, 1979), but they were not accepted universally. However, a recent approach to bacteriophage taxonomy, which is accepted universally, has identified three groups known as bacteriophage families, namely the *Myoviridae*, *Podoviridae* and *Siphoviridae* (Ackermann and DuBow, 1987; Francki *et al*., 1991). Bacteriophages of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* belong to the *Siphoviridae* family (Neve, 1996; Josephsen and Neve, 1998), and Fig. 6.3 illustrates an example of an isometric head structure of a bacteriophage of *S. thermophilus*. The overall morphology of bacteriophages of the yoghurt starter cultures are described as having an isometric head with non-contractile tails. Some bacteriophages may have a collar situated under the head and a base plate at the terminal tail structure including spikes (see Soldal and Langsrud, 1978). Bacteriophages are classified into two main categories depending on the growth responses in the bacterial host, and these types are virulant or lytic bacteriophages (i.e. those that can infect and lyse the host cell) and temperate, prophage or lysogenic bacteriophages (i.e. those that do not lyse the bacterial host, but instead insert their genome in the host chromosome) (Neve, 1996).

The lytic cycle of a bacteriophages involves several stages known as adsorption to the bacterial host, injection of bacteriophage DNA, bacteriophage maturation and lysis of the bacterial cell. The lysogenic cycle primarily involves only the first two stages since rather than the bacteriophage maturing in the bacterial host, the bacteriophage DNA is inserted into the bacterial chromosome. According to Neve (1996) and Josephsen and Neve (1998), this action occurs by a single reciprocal

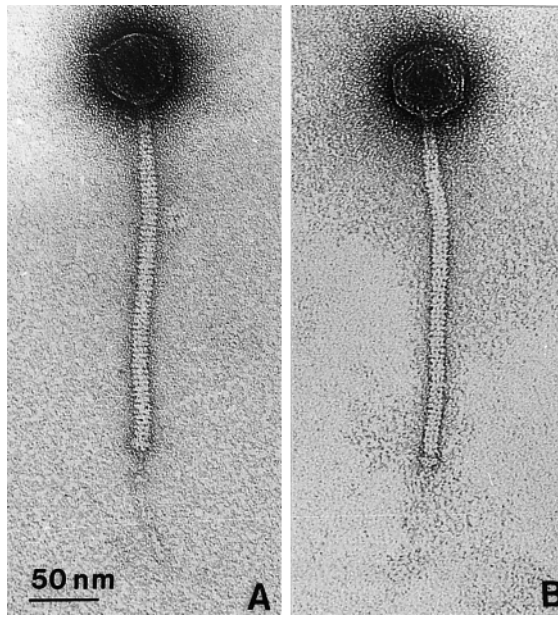

Fig. 6.3 Illustration of a transmission electron micrograph showing different morphological characteristics of a virulent bacteriophage of *S. thermophilus* with (A) and without (B) a tail fibre

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recombination event taking place at a specific region of homology between the bacteriophage DNA and the bacterial host DNA which is known as an attachment site (i.e. attP in the bacteriophage genome and attB in the bacterial host). Thus, bacterial host lysis does not occur and the bacteriophage DNA is now known as probacteriophage and is replicated simultaneously with bacterial host DNA, giving rise to a progeny of lysogenic cells. This bacteriophage is known as a temperate bacteriophage. Over the years, many researchers have used electron microscopy to observe the morphology of bacteriophages of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (see Table 6.7).

Accolas and Spillmann (1979a) observed that six out of seven *S. thermophilus* bacteriophages were similar, that is the head, which was polyhedral or possibly octahedral, was 49–53 nm in diameter, the tail length ranged from 200 to 224 nm (with the exception of one, i.e. 130 nm) and the tail width from 8 to 9 nm; the tail tip had a small plate covered with short prongs or a fibrous mass; the seventh type of phage had no specific tail-tip structure. However, a recent study by Krusch *et al.* (1987) suggested that streptococcal bacteriophages obtained from different research laboratories in Europe have different morphological sizes (see Table 6.7). The distinctive characteristics of *S. thermophilus* bacteriophages can be summarised as follows:

- The sensitivity of the organism to bacteriophage attack was described by Pette and Kooy (1952) under one of three headings: bacteriophage-insensitive, bacteriophage-tolerant (i.e. carriers of the particles) and bacteriophage-sensitive (i.e. results in complete lysis of the host cell).

Table 6.7 Morphology (range) of bacteriophages of yoghurt starter cultures

Micro-organism	Head		Tail	Tail Tip	(n =)[b]	References
	Structure	Size[a] (nm)	Length (nm) × diameter (nm)			
S. thermophilus	Hexagonal	50–60	217–239 × 4.8	–	2	Sarimo and Moksunen (1978)
	Polyhedron	40–60	220–420 × 8	–	2	Koroleva *et al*. (1978)
	Polyhedral or octahedron	49–50	130–224 × 8–9	+	14	Accolas and Spillmann (1979a)
	NR	60–65	236–290 × 10	+	3	Reinbold *et al*. (1982)
	Polyhedral	48–70	213–265 × 11–12	±	59	Krusch *et al*. (1987)
	Isometric	57	234 × 9.5 (mean)	+	50	Carminati *et al.* (1994)
	Hexagonal	45–65	220–245 × NR	+	120	Fayard *et al*. (1993)
	Isometric	65	230–260 × NR	+	24	Brüssow *et al*. (1994)
L. delbrueckii subsp. *bulgaricus*	Hexagonal	56–62	205–215 × NR	+	1	Peake and Stanley (1978)
	Polyhedral or octahedron	44–55	116–160 × 8–9	±	7	Accolas and Spillmann (1979b)
	NR	50–59.4	175–198 × 5–6.6	–	3	Reinbold *et al*. (1982)
	Hexagonal	47	159 × NR	–	1	Auad *et al*. (1997)

n = Number of strains tested. NR: not reported.

- A similar classification was proposed by Sarimo and Moksunen (1978), but they incorporated some morphological features as well. Russian workers (Koroleva *et al*., 1978) divided the bacteriophages of *S. thermophilus* into two groups based on morphological observations: regular polyhedron head 40 nm in diameter and others, i.e. head size 65 nm in diameter.
- All virulent bacteriophages of *S. thermophilus* belong to one DNA homology group (e.g. genome size 37–44 kb (Kivi *et al*., 1987; Neve *et al*., 1989; Larbi *et al*., 1990, 1992; Fayard *et al*., 1993; le Marrec *et al*., 1997) and based on the protein profiles and degree of homology of these bacteriophages, they were classified into two or three subgroups (see also Prevots *et al*., 1989; Benbadis *et al*., 1990; Sebastiani and Jäger 1992, 1993; Brüssow *et al*., 1994; Bruttin *et al*., 1997a, b).
- Larbi *et al*. (1992) identified three different mechanisms of bacteriophage resistance in the bacterial host, one of which exhibited a temperature-dependent response.
- Expression of a *Lac. lactis* subsp. *lactis* plasmid-encoded bacteriophage defence mechanism in *S. thermophilus* increased the bacteriophage resistance in the *Streptococcus* (Moineau *et al*., 1995).
- The conductance measurement technique and spot test method have been used successfully for bacteriophage detection in *S. thermophilus* and a yoghurt culture, respectively (Carminati and Neviani, 1991; Champagne and Gardner, 1995).
- Lysogenic strains and many temperate bacteriophages of *S. thermophilus* may have an endogenous origin (Carminati and Giraffa, 1992).

Virulent bacteriophages attacking *S. thermophilus* host cells result in lysis of the cell wall by an enzyme, lysin, which releases newly formed bacteriophages into the growth medium. A typical illustration of which can happen to such a culture before and after infection with a bacteriophage is shown in Fig. 6.4.

In the 1970s, some of the distinctive morphological features of *L. delbrueckii* subsp. *bulgaricus* bacteriophages were reported by Peake and Stanley (1978) and Accolas and Spillman (1979b) and, in brief, they are (a) shorter in overall length in comparison with *S. thermophilus* bacteriophages (e.g. 116–198 nm) with the exception of those phages studied by Peake and Stanley (1978), where the length varied from 205 to 215 nm, (b) the presence of a "collar" structure, and (c) the appearance of up to ten "cross-bar" structures intersecting the tail at intervals (see Table 6.7). More recent characterisations of the lactobacillar bacteriophages have included the following:

- Both lytic and temperate bacteriophages have been found in *L. delbrueckii* subsp. *bulgaricus* and subsp. *lactis*, and their classification has been reported by Cluzel *et al.* (1987a, b), Sechaud *et al*. (1988) and Lahbib-Mansais *et al*. (1988).
- A temperate bacteriophage infecting *L. delbreuckii* subsp. *bulgaricus* had a circularly permuted and terminally redundant genome with a unique sequencing of 36 kb, and was capable of infecting *L. delbrueckii* subsp. *lactis* (Boizet *et al*., 1990; Lahbib-Mansais *et al*., 1992; Auad *et al*., 1997); a virulent bacteriophage has a linear genome of 35 kb (Chow *et al*., 1988).
- Vescovo *et al*. (1990) reported on the sensitivity to bacteriophages of morphological variants of *L. delbrueckii* subsp. *bulgaricus* (e.g. curved or straight cells) and suggested that the physiological reactions were influenced by calcium and magnesium.

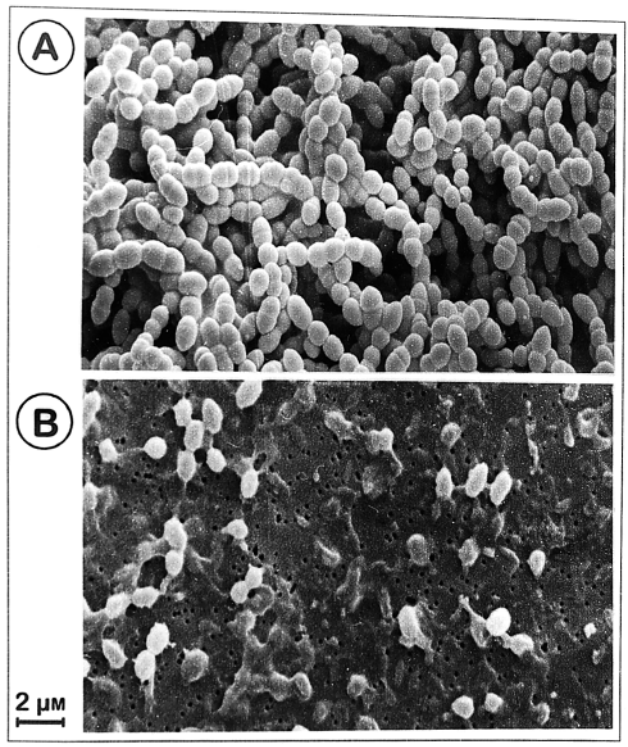

Fig. 6.4 Scanning electron micrograph illustrating (A) a healthy *S. thermophilus* culture and (B) the lysis of cells after infection with a virulent bacteriophage

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No data are available on infection of *L. acidophilus* and *Bifidobacterium* species with bacteriophages. It could be argued that the presence of these cultures in bio-yoghurt is for the provision of probiotic cells in the product rather than for the production of acid for the gellation of milk. However, the interest generated in using these organisms as starter cultures may initiate research work on their bacteriophages. Research work on the bacteriophages of thermophilic lactic starters has increased substantially since the 1970s and Table 6.7 reviews the morphology of those bacteriophages of the yoghurt organisms that have been reported in the literature up to the present time. Figure 6.5 shows some of the morphological characteristics of the bacteriophages that can infect *L. delbrueckii* subsp. *bulgaricus*.

It is also relevant, concerning the viruses attacking *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, that:

- If milk is the origin of bacteriophage contamination, then heat treatment at 85°C for 20 min ensures their destruction (Stolk, 1955); raw milk could be the source of bacteriophages, thus causing problems during the manufacture of some traditional cheeses from raw milk in Europe.
- The optimum temperature of bacteriophage proliferation is the same as the optimum growth temperature of the host, i.e. *S. thermophilus* phages at 39–40°C

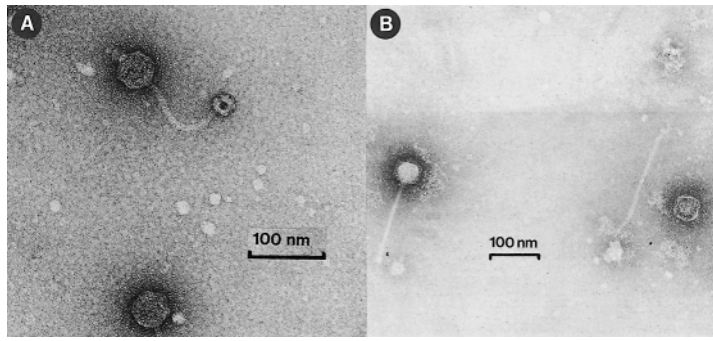

Fig. 6.5 Electron micrograph showing the morphology of two bacteriophages of *L. delbrueckii* subsp. *bulgaricus*

(A) Bacteriophage c5 of strain LT4; note the isometric head, non-contractile flexible and regularly striated tail and large basal plate. (B) Bacteriophage y5 of strain Y5; note the isometric head with large triangular "facets", a small fibrous collar and tail tip composed of short fibres.

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and *L. delbrueckii* subsp. *bulgaricus* bacteriophages at 42–43°C (Sozzi *et al*., 1978).

- Chemical sterilisation of equipment using 0.1% QAC, 70–90% ethanol, 0.5–1.0% potassium permanganate or 50–100 mg l^{-1} of available chlorine causes the destruction of *S. thermophilus* phages (Ciblis, 1966); peracetic acid (120–300 mg l^{-1}) and active chlorine (≥2.6 mg l^{-1}) were recommended by Langeveld and van Montfort-Quasig (1995, 1996) for inactivating yoghurt starter culture bacteriophages (see also Neve *et al*., 1996).
- Phages are species and/or strain specific, i.e. phages of mesophilic lactic starters do not attack thermophilic starter cultures, and furthermore, *S. thermophilus* phages do not attack *L. delbrueckii* subsp. *bulgaricus* strains.
- The lysis of *Lactobactillus* species including *L. delbrueckii* subsp. *bulgaricus* in the vagina was due to the action of bacteriocins produced by certain lactobacilli and bacteriophages (Tao *et al*., 1997).

It is evident, therefore, that one or more of the following precautionary measures should be practised in order to eliminate or control phage attack (see also IDF, 1991b):

- use of aseptic techniques for the propagation and production of starter cultures;
- ensure effective sterilisation of utensils and equipment;
- ensure proper heat treatment of the milk;
- restrict movement of plant personnel in starter handling room, and locate starter room far away from production area;
- check filtration of air into the starter room and production area;
- "fog" the atmosphere in the starter room with hypochlorite solution (not to be encouraged) or use laminar-flow cabinets for small-scale culture transfers;
- grow starter culture in bacteriophage inhibitory medium (BIM);
- use a daily rotation of bacteriophage unrelated strains (or phage-resistant strains) of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Havlova and Jicinska, 1985);

- produce the bulk starter culture or even the retail product using a direct-to-vat system;
- the use of turpine (obtained from aromatic plants by steam distillation) at a rate of 500 mg $100l^{-1}$ or black pepper oil inhibited bacteriophage infection but not the growth of *L. delbrueckii* subsp. *bulgaricus* (Wolf *et al*., 1983);
- growth of the yoghurt organisms in soy-milk stopped bacteriophage infection (Farhat *et al*., 1984).

6.3.9 Bacteriocins

Antibacterial substances (usually segregated from antibiotics) are produced by a wide range of bacteria including dairy starter cultures. They were termed colicin-like, but currently they are known as bacteriocins. For further information refer to the following reviews (Piard and Deamazeaud, 1992; Nettles and Barefoot, 1993; Barefoot and Nettles, 1993; Hoover and Steenson, 1993; de Vuyst and Vandamme, 1994; Nes *et al*., 1996; Marshall and Tamime, 1997). In general, Tagg *et al*. (1976) characterised bacteriocins as follows:

- proteinaceous in nature
- bactericidal rather than just bacteristatic
- capable of linking to specific binding sites on the bacterial cells and showing different activity from other antimicrobial substances
- plasmid-mediated
- Active against bacteria of the same genera.

At present, around 70 different types of bacteriocins have been identified and produced by lactic acid bacteria. Table 6.8 summarises some selected characteristics of the bacteriocins produced by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, and careful selection of the streptococci strains of the starter culture blend is important to minimise their inhibition. However, other lactic acid bacteria including *Propionibacterium* species can produce bacteriocins that are slightly inhibitory to *L. delbrueckii* subsp. *bulgaricus* (see Table 6.9). The use of such organisms beside the yoghurt starter culture is aimed at controlling over- or postacidification in the product (Weinbrenner *et al*., 1997). It could be of practical relevance that a bacteriocin produced by *S. thermophilus* affected the growth of *L. delbrueckii* subsp. *bulgaricus* only in M17 broth and not in milk (Cilano *et al*., 1991; see also Sikes and Hilton, 1987).

Limited data have been published on the mode of action of bacteriocins produced by lactic acid bacteria that can affect the yoghurt starter cultures. For example, lacticin B is bactericidal to sensitive cells, but it does not cause cellular lysis of host cells. It adsorbs non-specifically to sensitive and insensitive lactobacilli because it is a highly hydrophobic peptide and the mode of action may be similar to nisin and pediocin AcH (de Vuyst and Vandamme, 1994).

6.3.10 Miscellaneous factors

6.3.10.1 UF milk

The associative growth by *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was lower in ultrafiltered (UF) milk than in milk (Tayfour *et al*., 1981). A similar

Table 6.8 Some selected characteristics of bacteriocins produced by yoghurt starter cultures

Starter organisms and strain	Bacteriocin name	Molecular mass (kDa)	Sensitivity	Comments
S. thermophilus	NR	<0.7	NR	Antimicrobial compound is heat stable (100°C for 10 min) and and displayed inhibitory activity to Gram-negative and Gram-positive bacteria.
STB 40 and 78	STB 40 and 78	10–20	Lipase, α-chymotrypsin, trypsin and pronase	Both bacteriocins are stable between pH 2 and 12 and are heat resistant; they are active against *Enterococcus* spp. and *S. thermophilus* strains.
ST 10	ST 10	>100	Proteolytic enzymes and α-amylase	Only active against *S. thermophilus* and heat stable at 121°C for 15 min.
SFI 13	Thermophilin 13	~4.0	NR	Thermophilin is heat stable (100°C for 1 hour) and active in the pH range 1.6–10.
L. delbrueckii subsp. *bulgaricus* DDS 14	Bulgarican	NR	NR	Thermostable (120°C for 60 min) and only active at acidic pH; displayed a wide spectrum of inhibiting Gram-positive and Gram-negative bacteria.
7994	NR	<0.7	NR	Still active at pH 4 and thermostable for 1 hour at 100°C; it is active against *Pseudonomas* and *Staphylococcus* species.
CFR 2028	NR	NR	NR	Active principal of the bacteriocin is proteinaceous in nature; stable at pH 3.8–5.0 and heat for 75°C for 30 min; active against *Bacillus cereus*.

NR, not reported.

Data compiled from Abdel-Bar *et al*. (1987), Cilano *et al*. (1990, 1991), Marshall and Tamime (1997) and Balasubramanyam and Varadaraj (1998).

Table 6.9 Inhibition of yoghurt starter cultures by bacteriocins produced by different micro-organisms

Micro-organisms	Bacteriocin name/molecular mass (kDa)	Comments and references
L. delbrueckii subsp. *lactis*	Lacticin A & B kDa (NR)	Inhibited growth of *L. delbrueckii* subsp. *bulgaricus*[a] (Toba *et al*., 1991)
	Lactobacillin G4 kDa (NR)	As above (Giraffa *et al*. 1989, 1990)
	No name <1 kDa	As above (Hara *et al*., 1995)
Lac. lactis subsp. *lactis*	No name 1–10 kDa	As above (Su and Lin, 1990)
	Lacticin 481 1.3–2.9 kDa	As above (Piard and Desmazeaud, 1992)
L. acidophilus	Acidophilucin A kDa (NR)	As above (de Vuyst and Vandamme, 1994)
	Lactacin B 6.2–8.1 kDa	
	Lactacin F 2.5–6.3 kDa	
L. helveticus	Helveticin J 37 kDa	
Propionibacterium jensenii	Jenseniin G	As above (Weinbrenner *et al*., 1997)
Lactobacillus reuteri	Reutericin 6 2.7 kDa	As above (Kabuki *et al*., 1996)
Lac. lactis subsp. *lactis*	Lactococcin DR 2.3–2.4 kDa	Inhibited growth of *S. thermophilus* (de Vuyst and Vandamme, 1994)
	Lacticin 481 1.3–2.9 kDa	

NR, not reported.

[a] Lacticin A is active against this micro-organism.

observation was recently reported by Radulovic and Obradovic (1997), but they observed that the lactobacilli showed better acid development than the streptococci. Ozen and Ozilgen (1992) reported that the kinetic analysis clearly illustrated that the contribution of each microbial species of the yoghurt organisms to the mixed culture growth changed drastically when the substrate concentration was about 15 g 100 g^{-1}.

6.3.10.2 Added flavours

The addition of coffee (*Coffee robusta*) extract, ginseng saponins and garlic extract to the milk base before fermentation reduced acid development during the manufacture of yoghurt, dahi and acidophilus milk, or in milk inoculated with single strains of lactic acid bacteria (Kim *et al*., 1987; Gandhi and Ghodekar, 1988; Fardiaz, 1995).

6.3.10.3 Lysozyme

This compound is sometimes added to cheese milk to control or inhibit the growth of clostridia. Most of the *L. helveticus* strains have been found to be sensitive to

lysozyme at low concentrations of 10 or 20 $\mu g\,ml^{-1}$, but not the yoghurt starter organisms (Neviani *et al*., 1988a). However, a strain of *L. delbrueckii* subsp. *bulgaricus* that was sensitive to lysozyme was cultured eight times in the presence of 100 $\mu g\,g^{-1}$ of lysozyme; it developed some resistance but lost it on subsequent culturing in milk (Neviani *et al*., 1988b); lysozyme resistance is thought to be plasmid related (see also Mercenier *et al*., 1988a, b).

6.3.10.4 Diet of the cow

At certain times of the year (i.e. June to August in Italy), the acidification rate of the yoghurt organisms is reduced, but activity is retained when the milk is supplemented with paraffin, vitamin E or Fe^{2+} and Zn^{2+}; the problem may also be reduced by supplementing the cow's diet with vitamins (Maianti *et al*., 1996).

6.3.10.5 Nitrates ($NaNO_3$) and nitrites ($NaNO_2$)

The presence of nitrites in some dairy products is permitted at a level of 0.01 mg 100 ml^{-1} (Baranova *et al*., 1997). However, the addition of the nitrates or nitrites to the milk base reduced the rate of acid development by yoghurt cultures (Korenekova *et al*., 1997; Baranova *et al*., 1997) and the resulting products had low viscosities. Changes in the $NaNO_3$ content in yoghurt, including interactions with caseins, have been reported by Steinka and Przyblowski (1994, 1997).

6.3.10.6 Radioactive materials (^{131}I)

Contamination of milk with such components is undesirable, but in view of the Chernobyl accident, Greek scientists studied the effect of adding ^{131}I to milk during the manufacture of yoghurt and labneh (Vosniakos *et al*., 1991, 1992, 1993; see also Section 5.7 in Chapter 5; Micic *et al*., 1985). An ^{131}I content in milk amounting to 6–12 $kBq\,kg^{-1}$ reduced the counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* by 45–52% in set yoghurt and labneh; lactococcal species were reduced by 30% in cheese and buttermilk and 26% in ripened butter.

6.3.10.7 Aflatoxins

Aspergillus flavus and *parasiticus* have been identified as producing toxins ($AFB_{1\&2}$ and $AFG_{1\&2}$) that have been implicated as acute toxicants and heptacarcinogens in the human (El-Nezami and Ahokas, 1998). Their presence in yoghurt is discussed in Chapter 10, but research work regarding the role of lactic acid bacteria in controlling the growth of *Aspergillus* species is very limited. However, certain mesophilic and thermophilic starter cultures are capable of detoxifying aflatoxin (El-Nezami and Ahokas, 1998).

Mohran *et al*. (1985) showed that whereas AFB, added to skimmed milk (up to 0.44 $\mu g\,ml^{-1}$), did not affect the growth of *S. thermophilus* and lactococcal species, *L. delbrueckii* subsp. *bulgaricus* and *L. paracasei* subsp. *paracasei* were inhibited, but Kalra *et al*. (1977) observed the opposite effect on the yoghurt organisms; the yoghurt starter cultures were very effective in the detoxification of 0.5 $mg\,l^{-1}$ ochratoxin A present in milk (Rasic *et al*., 1991; Skrinjar *et al*., 1996).

6.3.10.8 Sweetening agents

The addition of sugar $\geq 9\,g\,100\,g^{-1}$ to the milk may cause inhibition or delay in the fermentation period, as will the addition of artificial sweetners. For further details refer to section 2.6 in Chapter 2 (see also Lacroix and Lachance, 1988a, b, 1990; Larsen and Anon, 1989a, 1990; Latrille *et al*., 1992).

6.3.10.9 Cadmium (Cd)
As a result of environmental pollution, Cd may be found in cow's milk at low levels up to 160 μg kg^{-1} with typical values <0.5 μg kg^{-1} (Walstra and Jenness, 1984). An inhibition of the decrease in pH was observed for *S. thermophilus* >5 μg Cd l^{-1} (Korkeala *et al*., 1984), but not at lower levels.

6.3.10.10 Phosphates
Bacteriophage inhibitory media (BIM) for lactococci contain high levels of phosphates which chemically bind the free calcium in milk, thus preventing bacteriophage replication (Zottola and Marth, 1966). However, the growth of *L. delbrueckii* subsp. *bulgaricus* in phosphated milk (i.e. added phosphate or commercially available BIM) was inhibited and cellular morphology was altered in milk containing about 3 g 100 g^{-1} phosphate (Wright and Klaenhammer, 1983, 1984). Shalaby *et al*. (1986) observed no effect on growth of four strains of *S. thermophilus* in phosphated media and when milk containing sodium citrate + sodium phosphate, yeast extract and infected with bacteriophage was used, the rate of acid production was not reduced either; the presence of the buffering agents was effective in suppressing bacteriophage attack. However, Champagne and Gange (1987) observed that the starter activity of three strains of *S. thermophilus* growth in Phase 4 and In-sure (i.e. a commercially available BIM) was influenced by two factors: (a) the age of the culture, for example, the starter cultures lost their activity in milk after 16–24 hour, whilst in BIM retained their activity for 40–48 hours, and (b) the heat treatment used for preparation of the BIM and agitation during growth affected *S. thermophilus* activity in relation to the BIM used (i.e. In-sure but not Phase 4).

6.3.10.11 Preservatives
In some countries, the addition of preservatives (e.g. K- or Na-sorbate, benzoic acid or nisin) is permitted in fruit yoghurt, but not in natural yoghurt (for details refer to Section 2.7.2 in Chapter 2). These compounds are mycostatic agents and, at the same time, they can affect the activity of the starter cultures (see Table 2.12; Gupta and Prasad, 1988; Kebary and Kamaly, 1991; Rajmohan and Prasad, 1994).

6.3.10.12 Miscellaneous compounds
The concentration (mg l^{-1}) of fatty acids (1000), ethylenedichloride and methylsuphone (10–100 each) and acetonitrile, chloroform or ether (10 each) had an inhibitory effect on *S. thermophilus* (see also Tamime and Deeth, 1980; Antonopoulou *et al*., 1996).

6.4 Conclusion

It is evident that milk is an excellent growth medium for yoghurt starter cultures, but the rate of growth in milk is influenced by a multitude of factors. Thus, using milk free from these inhibitory agents, providing hygienic standards during the preparation of starter culture and production of yoghurt and using the right combination of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* will lead to successful growth. Future development of these cultures in terms of resistance against bacteriophage and/or inhibitory agents will help to minimise culture failure during production.

6.5 References

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